Plant Cell Rep (2006) 25: 223–230
DOI 10.1007/s00299-005-0073-6

GENETICS AND GENOMICS

**Kwang-Hyun Baek · Daniel Z. Skinner**

# Differential expression of manganese superoxide dismutase sequence variants in near isogenic lines of wheat during cold acclimation

Received: 2 May 2005 / Revised: 29 September 2005 / Accepted: 2 October 2005 / Published online: 25 November 2005


**Abstract** Numerous sequence variants of wheat (*Triticum aestivum* L.) manganese superoxide dismutase (MnSOD) genes have been found. Quantitative real-time PCR was used to measure the expression levels of three MnSOD genes distinguished by a variable amino acid, and three genes distinguished by sequence variation in the 3′ untranslated region (3′ UTR), in wheat plants grown at 20°C and cold-acclimated for 1–4 weeks at 2°C. The amino acid variants did not differ significantly in expression levels, however, differential expression of genes differing in the 3′ UTR was observed. Diploid wheat-related species also carried sequence variants of MnSOD, with differing levels of expression, suggesting diversification of the MnSOD gene family occurred prior to the polyploidization events of hexaploid wheat.

**Keywords** Cold acclimation · Manganese superoxide dismutase · Multigene family · Quantitative real-time PCR · Wheat

**Abbreviations** MnSOD: Mitochondrial manganese-superoxide dismutase · NIL: Near isogenic line · qRT-PCR: Quantitative real-time PCR · ROS: Reactive oxygen species · 3′ UTR: 3′ Untranslated region

## Introduction

Reactive oxygen species (ROS) such as hydrogen peroxide ($H_2O_2$), superoxide ($O_2^-$), and the hydroxyl radical ($OH^\bullet$) can be very harmful to living organisms because of their oxidizing potentials within living cells (Bowler et al. 1992). Although ROS are generated on a regular basis in biological pathways as by-products or signal transducers (Wu et al. 1997; Zelko et al. 2002), certain conditions can induce oxidative stress in plants, a state in which ROS accumulate to injurious or lethal levels due to excessive production or inefficient scavenging by antioxidant systems (Monk et al. 1989; Fridovich 1991). ROS can attack all the basic molecules of living organisms, such as lipids, carbohydrates, proteins, and nucleic acids, resulting in the peroxidation of membranes (Kendall and McKersie 1989), breakage of polysaccharides (Sato et al. 1993), inactivation of enzymes (Fucci et al. 1983), and "nicking," cross-linkage, and scission of DNA strands (Halliwell and Gutteridge 1999).

Plants have evolved very efficient antioxidant enzyme systems to scavenge ROS and protect from injury due to the oxidative stress (Bowler et al. 1991; Allen 1995). The superoxide dismutases (SODs) form one of the most effective antioxidant enzyme systems in plants. SODs (EC 1.15.1.1) dismutate two superoxide anions ($O_2^-$) into $H_2O_2$ and $O_2$ (Fridovich 1991). SODs comprise several isozymes, which can be classified by cellular location and the catalytic metals required; manganese superoxide dismutase (MnSOD) in mitochondria, iron-superoxide dismutase (FeSOD) in chloroplasts, and copper/zinc superoxide dismutase (CuZnSOD) in chloroplasts and in the cytosol (Fink and Scandalios 2002). Among these SODs, MnSOD is the only form of SOD that has been shown to be essential for the survival of aerobic life (Carlioz and Touati 1986).

Exposure to environmental stresses can stimulate enhanced ROS scavenging systems in plants, enhancing stress tolerance (Allen 1995). The increased scavenging activity of antioxidant enzymes may require de novo synthesis, initiated by upregulation of genes encoding antioxidant enzymes. The MnSOD enzyme in wheat is encoded by nuclear genes, but is the primary protective enzyme for oxidative stress in mitochondria. The expression of MnSOD genes in wheat significantly increased in response to cold temperature (Baek and Skinner 2003), suggesting an

Communicated by I. S. Chung

K.-H. Baek
Department of Crop and Soil Sciences, 210 Johnson Hall,
Washington State University,
Pullman, WA 99164-6420, USA

D. Z. Skinner (✉)
USDA/ARS, Department of Crop and Soil Science, 209
Johnson Hall, Washington State University,
Pullman, WA 99164-6420, USA
e-mail: dzs@wsu.edu
Tel.: +1-509-335-8696
Fax: +1-509-335-2553

increased level of antioxidant activity in the mitochondria in response to cold temperature. Fink and Scandalios (2002) suggested that the structural complexity of the mitochondrion necessitates the occurrence of MnSOD holoenzyme at several locations within mitochondria, because oxyradicals generated by oxidative phosphorylation in mitochondria cannot diffuse through the membrane lipid bilayers of mitochondrial subcompartments. Perhaps various MnSOD forms, such as we describe here in wheat and have been described in maize (Zhu and Scandalios 1993) and Hevea (Miao and Gaynor 1993), occur at different locations within the mitochondria.

Several studies have indicated that an increase in the expression of MnSOD enhances tolerance to many environmental stresses (Allen 1995). *Chlorella ellipsoidea* with a high amount of MnSOD showed enhanced tolerance to chilling stress (Clare et al. 1984). Chilling stress tolerance of *Zea mays* increased when a tobacco MnSOD gene was expressed in the chloroplasts (Breusegem et al. 1999). Both freezing tolerance and recovery from freezing stress were enhanced in *Medicago sativa* with a tobacco MnSOD gene expressed in the chloroplasts or mitochondria (McKersie et al. 1993). MnSOD transcripts increased significantly in both spring and winter wheat seedlings exposed to 2°C, suggesting an increased level of MnSOD enzyme was present, presumably affording increased protection of mitochondria from ROS injury (Wu et al. 1999; Baek and Skinner 2003). Overproduction of MnSOD in tobacco significantly increased tolerance of oxidative stress (Bowler et al. 1991). Transgenic *Brassica* plants overexpressing wheat MnSOD were more tolerant of oxidative stress and aluminum toxicity (Gachon et al. 2004). All of these data strongly suggest that increased expression of MnSOD in plants enhances tolerance of environmental stresses.

Considering that MnSOD is essential to living organisms (Carlioz and Touati 1986), at least one copy of MnSOD is expected in each plant genome. The hexaploid *T. aestivum* genome is comprised of genomes from three ancestral diploid species and therefore is expected to have at least three homeologous copies of MnSOD genes. MnSOD genes in wheat have been found on the long arm of the homeologous group-2 chromosomes (Wu et al. 1999), and mapping of wheat ESTs at GrainGenes (http://wheat.pw.usda.gov/wEST/) revealed five loci on group-2 chromosomes, suggesting there are paralogous MnSOD genes present in the wheat genome. Prior to the study reported here, three MnSOD cDNAs from wheat were available in GenBank: MnSOD (AF092524), MnSOD3.1 (U72212), and MnSOD3.2 (U73172). Based on the alignment of these three MnSOD genes, it appeared that MnSOD genes in wheat comprised a multigene family with members differing in the coding sequences and in the 3′ UTRs. We refer to these multiple genes as sequence variants, which may include both paralogous and homeologous forms.

It is known that expression of wheat MnSOD genes increased following exposure to low temperature (Wu et al. 1999; Baek and Skinner 2003), but whether each member of the MnSOD multigene family responded equally to the low temperature is unknown. The objectives of this study were to identify sequence variants of MnSOD genes and investigate differences in expression levels of the sequence variants in response to low temperature.

## Materials and methods

### Plant growth and low-temperature treatment

We used near isogenic lines (NILs) differing only at the *Vrn1-Fr1* (vernalization, frost tolerance) locus on chromosome 5A, which determines winter versus spring wheat growth habit, i.e., wheat with or without a vernalization requirement, respectively. NIL 442 is a winter wheat with genotype *vrn1vrn1Fr1Fr1* and NIL 443 is a spring wheat with genotype *Vrn1Vrn1fr1fr1* (Storlie et al. 1998). *Aegilops speltoides* (PI 573452), *Aegilops tauschii* (PI 603254), *Triticum monococum* subsp. *monococcum* (PI 596286), and *Triticum turgidum* subsp. *turgidum* (PI 347137) were provided by the USDA-ARS, National Small Grains Research Facility, Aberdeen, ID (http://www.ars.usda.gov/main/docs.htm?docid=2884). Seeds were planted in a commercial soiless potting mix and grown for 14 days in a growth chamber maintained at 20°C under 200 μmol quanta $m^{-2}$ $s^{-1}$, 16 h photoperiod. The temperature then was reduced to 2°C while light intensity and photoperiod were maintained. Whole leaves were collected approximately 4 cm above the crowns after 0, 7, 14, 21, and 28 days of cold treatment, frozen in liquid nitrogen, and total RNA was extracted as below.

### RNA and DNA extraction

The leaves collected from each individual plant were ground in liquid nitrogen with a mortar and pestle. Two milliliters of Trizol solution (Invitrogen, San Diego, CA, http://www.invitrogen.com) were added to the mortar before thawing of the plant material, and total RNA was extracted following the manufacturer's instructions. Extracted total RNA was quantified with a spectrophotometer (Jasco V-530, Jasco Inc., MD, USA, http://www.jascoinc.com). The RNA quality was assessed by running 2 μg of the total RNA on a 1.2% agarose gel. The total RNA was stored at −80°C.

DNA was extracted from leaves of NIL 442 and 443 using the method described by Dellaporta et al. (1985), followed by phenol–chloroform purification and ethanol precipitation.

### Alignment of MnSOD wheat genes and construction of plasmid clones the variant forms

DNA sequences of three wheat MnSOD genes (GenBank accessions AF092524, U72212, and U73172) were aligned using MultAlin (Corpet 1988; http://prodes.

toulouse.inra.fr/multalin/multalin.html; Fig. 1a and b). Nucleotide variation was found encoding amino acid position 166 (Fig. 1a) and in the 3′ UTR (Fig. 1b). Accessions AF092524, U72212, and U73172 had glycine, arginine, and lysine at position 166, respectively. We cloned this variable region from cDNA as follows. Total RNA from NIL 442 was used as template for cDNA construction using Thermoscript RT-PCR (Invitrogen, San Diego, CA, http://www.invitrogen.com) using oligo dT (20) primers according to the manufacturer's instructions. The resulting cDNA was used as the template for PCR. A 418 bp portion of the coding region including the encoded variable amino acid was amplified using forward primer 5′-CTACGTCGCCAACTACAACAAG and reverse primer 5′-GTAGTACGCATGCTCCCAGAC. PCR products were analyzed on 1.2% agarose gel and the amplified fragments were purified with GeneClean II kit (Q-Biogene, Carlsbad, CA, http://www.qbiogene.com). The purified PCR products were cloned using Topo-XL PCR cloning kit (Invitrogen, San Diego, CA, http://www.invitrogen.com), and the plasmid clones were sequenced at the DNA sequencing facility at Washington State University, Pullman, to confirm the cloned fragments were indeed parts of MnSOD genes, and to seek variant forms. The arginine and lysine forms were found in the PCR products, confirming variation was present in line 442. Similarly, variation in the 3′ UTR was found in this wheat line from PCR products amplified using forward primer 5′-CGTCCGCCGTCGTCCA and reverse primer 5′-AACAGCACTAGCGAACGAGTT. Sequence information from these products was then used to design primers to specifically amplify each form of the amino acid variants and the 3′ UTRs for quantitative real-time PCR (qRT-PCR).

## qRT-PCR conditions and analysis of data

The HPLC-purified primers for detecting the amino acid variants at position 166 were:

5′ forward primer: 5′-GGCCATTGATGAGGATTT TGG, paired with specific 3′ reverse primers:

1. 5′-GGAGTAGTTTCAACTGAAAGCCC (glycine (G) at position 166 detection);

(a)

```
           1       10        20        30        40        50        60        70        80
           |--------+---------+---------+---------+---------+---------+---------+---------|
MnSOD      MALRTLAAKKTLGLALGGAR------GVATFTLPDLPYDYGALEPAVSGEIMRLHHQKHHATYVAHYNKALEQLDAAVSK
MnSOD3.1   MALRTLAAKKTLGLALGGARPPAAARGVATFTLPDLPYDYGALEPAVSGEIMRLHHQKHHATYVANYNKALEQLDAAVSK
MnSOD3.2   MALRTLAAKKTLGLALGGARPLAAARGVATFTLPDLPYDFGALEPAVSGEIMRLHHQKHHATYVANYNKALEQLDAAVSK
Consensus  MALRTLAAKKTLGLALGGARp.aaarGVATFTLPDLPYD%GALEPAVSGEIMRLHHQKHHATYVAnYNKALEQLDAAVSK

           81      90        100       110       120       130       140       150       160
           |--------+---------+---------+---------+---------+---------+---------+---------|
MnSOD      GDASAYVHLQSAIKFNGGGHVNHSIFWKNLKPISEGGGEAPHGKLGWAIDEDFGSIEKLIKKMNAEGA-LQGSGWVWLAL
MnSOD3.1   GDASAYVHLQSAIKFNGGGHVNHSIFWKNLKPISEGGGEAPHGKLGWAIDEDFGSIEKLIKKMNAEGAALQGSGWVWLAL
MnSOD3.2   GDASAYVHLQSAIKFNGGGHVNHSIFWKNLKPISEGGGEPPHGKLGWAIDEDFGSIEKLIKKMNAEGAALQGSGWVWLAL
Consensus  GDASAYVHLQSAIKFNGGGHVNHSIFWKNLKPISEGGGEaPHGKLGWAIDEDFGSIEKLIKKMNAEGAaLQGSGWVWLAL

           161     170       180       190       200       210       220       231
           |--------+---------+---------+---------+---------+---------+---------+|
MnSOD      DKEAKGLSVETTPNQDPLVTKGSNLHPLLGIDVWEHAYYLQYKNVRPDYLTNIWKVVNWKYAGEEYEKVLA
MnSOD3.1   DKEAKRLSVETTPNQDPLVTKGSNLHPLLGIDVWEHAYYLQYKNVRPDYLTNIWKVVNWKYAGEEYEKVLA
MnSOD3.2   DKEAKKLSVETTPNQDPLVTKGSNLYPLLGIDVWEHAYYLQYKNVRPDYLTNIWKVVNWKYAGEEYEKVLA
Consensus  DKEAK.LSVETTPNQDPLVTKGSNLhPLLGIDVWEHAYYLQYKNVRPDYLTNIWKVVNWKYAGEEYEKVLA
```

(b)

```
           1       10        20        30        40        50        60        70        80
           |--------+---------+---------+---------+---------+---------+---------+---------|
MnSOD      TTTGTCTGATGAGTAGACAGACAGTACTTGGCTCGCTCTTGAGTTTCCCGCTGTTGGATCTTTGTATATGCAGTAATAAA
MnSOD3.1   TTTGTCCGA---GTAGACAGACAGTACTTCGCTCGCTCTTGAGTTTCCCGCTGTTGGATCTTTGTATATGCAGTAATAAA
MnSOD3.2   TTTGTCTGATGAGTAGACAGACGGTACTTTGCTCGCTCTTGAGTTT----------------GTATATGCAGTAATAAA
Consensus  TTTGTCtGAtgaGTAGACAGACaGTACTT.GCTCGCTCTTGAGTTTcccgctgttggatctttGTATATGCAGTAATAAA

           81      90        100       110       120       130       140       150       160
           |--------+---------+---------+---------+---------+---------+---------+---------|
MnSOD      AGTGGGCACTATTCCACTGTCGGGACCTTCTGTACATTGCATAGAGGTGACACTACTGTCTAAAGCGCA--CTAGTGCAT
MnSOD3.1   AGTGGGCACTATTCCACTGTCGGGACCTTCTGTACATCGCATAGAGGCGGGG-TAATGTCCAACGCATAGAACAGTGCCT
MnSOD3.2   AGTGGGCACTATTCCACTGCCGGGATCGTCTGTACATTGCATAGAGGTGG-ACTACTGTCTAATGCGCACTACAGTGCCT
Consensus  AGTGGGCACTATTCCACTGtCGGGAcCtTCTGTACATtGCATAGAGGtGg.acTAcTGTCtAA.GCgcA..acAGTGCcT

           161     170       180       190       200       210       22022
           |--------+---------+---------+---------+---------+---------+-|
MnSOD      GTCCATTTTGGTACTTGTTACTGTGCCTGC-GTTGCTAATAAACTCATTCGCTAGTGCTGTT
MnSOD3.1   GTCCATTTTGGTACTTTTTACTGTGCCTGC-GTGACTAATAAACTCGTTCGCTAGTGCTGTT
MnSOD3.2   GTCCATTTTGGTACTTGTTACTGTGCCTGCTGTGGCTAATAAA
Consensus  GTCCATTTTGGTACTTgTTACTGTGCCTGC.GTggCTAATAAActc.ttcgctagtgctgtt
```

**Fig. 1** **a** Alignment of amino acid sequences of wheat MnSOD. The three MnSODs differ at position 166 (amino acids G, R, and K). **b** Alignment of 3′ UTR DNA sequences of wheat MnSOD

2. 5′-TTAGGAGTAGTTTCAACTGAAAGCT (lysine (K) detection);
3. 5′-GGAGTAGTTTCAACTGAAAGCCT (arginine (R) detection).

The HPLC-purified primers used for detecting 3′ UTR variants were based on Fig. 1b; 5′ forward:

1. 5′-GCRTGATTTGTCYGATGA (bases 10–12 present),
2. 5′-GCRTGATTTGTCYGAGTA (bases 10–12 absent);

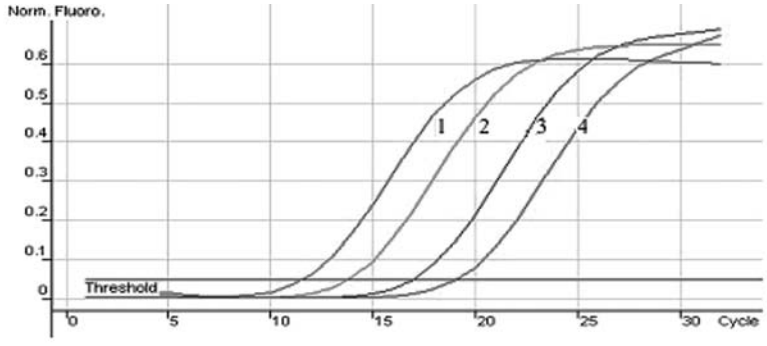
3′ reverse:

1. 5′-TTTTATTACTGCATRTACAAAGAT (bases 47–63 present);
2. 5′-TTTATTACTGCATRTACAAACTC (bases 47–63 absent).

The oligonucleotides were designed to have closely matched melting temperatures by calculating Tm with IDT Oligoanalyzer 3.0 (http://207.32.43.70/biotools/oligocalc/oligocalc.asp).

qRT-PCR was carried out on a RotorGene 2000 unit (Corbett Research, Sydney, Australia, http://www.corbettresearch.com) using SYBR green (product number S7567; Molecular Probes, Eugene, OR, http://probes.invitrogen.com) for detection of the product at the end of each amplification cycle (Bustin 2000; Karsai et al. 2002). qRT-PCR was carried out using Superscript One-step RT-PCR kit with Platinum Taq (Invitrogen, San Diego, CA, http://www.invitrogen.com). The cDNA construction and PCR amplification were performed in a single tube. The process consisted of cDNA construction for 15 min at 50°C followed by a PCR profile of a 3 min denaturation at 95°C, then 32 cycles of 15 s at 95°C, 20 s at 54°C, and 17 s at 72°C. The qRT-PCR solution was composed of 1× reaction buffer from the Superscript One-step RT-PCR kit, 2 mM $MgCl_2$, 1 μM primers, 1:40,000 SYBR Green, and 125 ng total RNA in 20 μl reactions. The reaction solution was covered with 9 μl light mineral oil (Sigma, St. Louis, MO, http://www.sigmaaldrich.com) to prevent evaporation during qRT-PCR. All qRT-PCRs were repeated three times as completely independent replications. We used known concentrations of cloned MnSOD double-stranded DNA to generate data for a standard curve. Melting curves of the final qRT-PCR products were generated to confirm a single PCR product had been formed. We evaluated the efficiency with which the primers discriminated among the targets of qRT-PCR by running PCRs using plasmid clones differing at amino acid position 166 or in the 3′ UTR as template. The expression data for the transcript copy numbers were analyzed by Duncan's means separation available in PROC ANOVA of the software SAS Version 6.12 (SAS Inc., Cary, NC, http://www.sas.com/).

qRT-PCR for quantifying the genomic 3′ UTR variants of MnSOD was performed using Taq polymerase (Promega, Madison, WI, http://www.promega.com). qRT-PCR amplification conditions were: 3 min denaturation at 95°C, then 32 cycles of 20 s at 95°C, 30 s at 54°C, and 30 s at 72°C. The qRT-PCR reaction solution was composed of 50 mM KCl, 10 mM Tris–HCl, 0.1% Triton X-100, 1.7 mM $MgCl_2$, 1 μM primers, 1:40,000 SYBR Green, 200 μM dNTPs, and




| Name of reaction | Amino acid in position 166 | Line number on the graph | Amplified product reached threshold at cycle number: | Experimentally Determined copy number |
|---|---|---|---|---|
| K1 | K | 1 | 11.57 | 521,148 |
| K0.2 | K | 2 | 13.8 | 116,114 |
| G1 | G | 3 | 16.95 | 13,925 |
| R1 | R | 4 | 19.08 | 3,319 |

**Fig. 2** Graph of qRT-PCR using plasmids differing at amino acid position 166 as template. The HPLC-purified primer set was: forward 5′-GGCCATTGATGAGGATTTTGG and reverse: 5′-TTAGGAGTAGTTTCAACTGAAAGCT, designed to match the K form at position 166. Reaction K1, G1, and R1 had the same amount of the respective cloned gene as templates. Reaction K0.2 had 20% of the same template as in reaction K1. Amplification results showed the specific primer amplified the corresponding plasmid much more efficiently than the other plasmid templates, and the calculated target number in reaction K0.2 very closely approximated the 20% reduction in target number

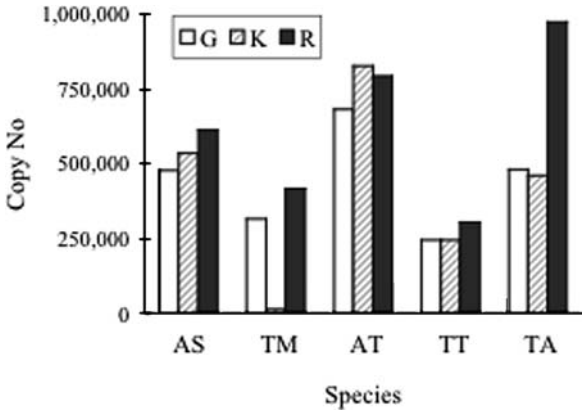



**Fig. 3** qRT-PCR determination of the transcript numbers of MnSOD variants with amino acid G, K, or R at position 166 in wheat-related species. AS: *A. speltoides*; TM: *T. monococcum*; AT: *A. tauschii*; TT: *T. turgidum*; TA: *T. aestivum* NIL 442. All accessions were grown at 20°C

300 ng total DNA in 25 μl reactions covered by 9 μl of mineral oil.

## Results

### Differential expression of amino acid position 166 in wheat-related species

The different amino acid variants at position 166 were named the G (glycine), K (lysine), or R (arginine) form following the amino acid present at position 166 (Fig. 1a). The HPLC-purified primer sets specifically discriminated the cloned G, K, or R forms with qRT-PCR very well (example in Fig. 2), therefore, qRT-PCR could be used to quantify the occurrence of the G, K, or R forms. Three-week-old plants of wheat-related species were examined to assess the expression levels of the variants at amino acid position 166 (Fig. 3). The data showed that wheat-related species have more than one copy of MnSOD, suggesting that these relatives had multigene families of MnSOD even before tetraploidization (*T. turgidum*) or hexaploidization (*T. aestivum*, Fig. 3). Interestingly, there was little or none of the K form transcript in *Triticum monococcum*, suggesting that *T. monococcum* may have two MnSOD genes (G or R at amino acid position 166) while other wheat-related species, e.g., *A. speltoides* and *A. tauschii*, have at least three MnSOD genes in the genome (Fig. 3).

### Differential expression of genes varying at amino acid position 166 in NIL 442 and 443 exposed to cold temperature

The expression levels of MnSOD genes varying at amino acid position 166 were measured in cold-treated NIL 442 and 443 (Fig. 4). All position 166 variants (G, K, and R forms) were expressed at 20°C (week 0), and significantly increased expression levels after exposure to cold temperature in both NIL 442 and 443. Expression levels reached a maximum at 3 weeks of cold treatment, and then declined after 4 weeks (Fig. 4). The numbers of copies of each of the transcript forms increased about five-fold after 3 weeks of cold acclimation. There were no significant differences in the expression levels of each form at a given week between NIL 442 and 443. The R form expressed the most among the three forms in normal growth conditions and cold acclimating conditions.

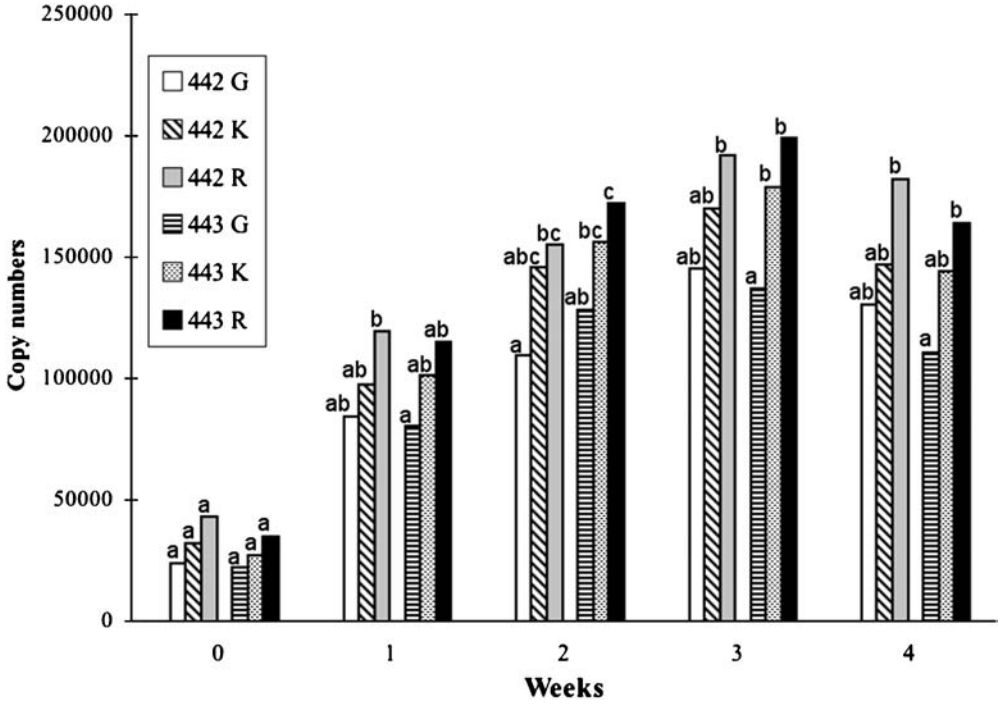



**Fig. 4** qRT-PCR determination of the numbers of copies of transcripts of MnSOD genes varying at amino acid position 166 after 0, 1, 2, 3, or 4 weeks of cold acclimation of wheat NIL 442 and 443. Copy numbers reported are per ng of total RNA. Bars with the same letter were not significantly different according to Duncan's means separation, $p=0.05$. Comparisons were made only within weeks

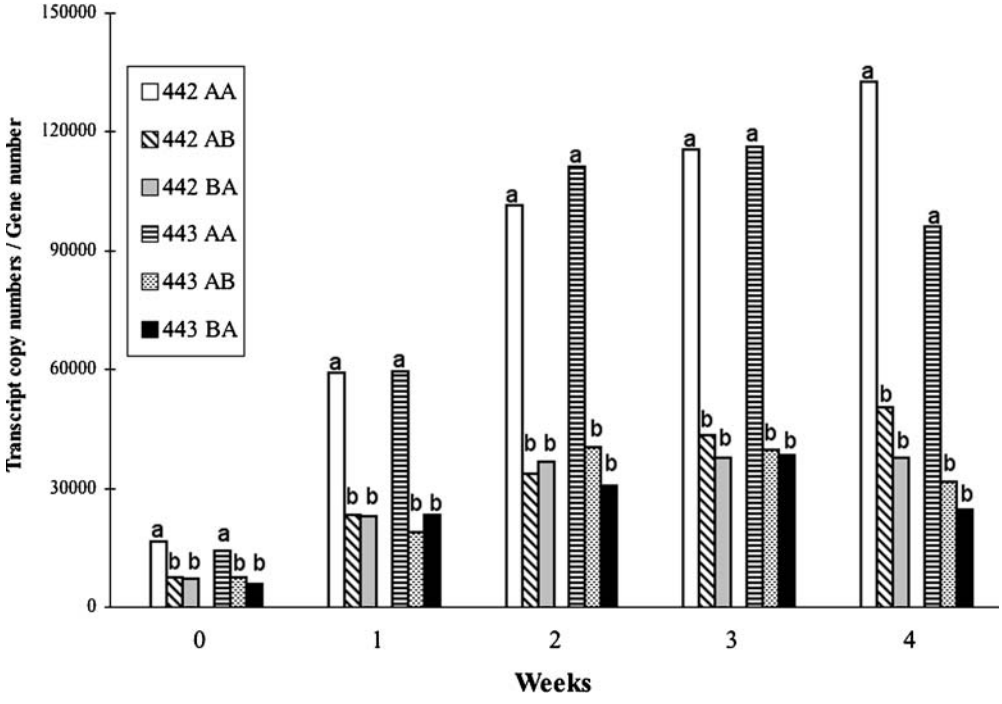



**Fig. 5** Copy numbers of 3′ UTR variant mRNA transcripts of MnSOD in wheat NIL 442 and 443 expressed as number of copies per ng total RNA divided by the number of copies of the corresponding genes in 1 ng of total DNA (approximately mRNA copies per gene) over 4 weeks of cold acclimation. AA variant: bases 10–12 present, and bases 47–63 present; defined in Fig. 1b; AB variant: bases 10–12 present, and bases 47–63 absent; BA variant: bases 10–12 absent, and bases 47–63 present

Differential expression of 3′ UTR variant forms

The wheat MnSOD genes varying in the 3′ UTR were designated as AA (bases 10–12 present, and bases 47–63 present; Fig. 1b), AB (bases 10–12 present, and bases 47–63 absent; Fig. 1b), BA (bases 10–12 absent, and bases 47–63 present; Fig. 1b), and BB (bases 10–12 absent, and bases 47–63 absent). The HPLC-purified primer sets were first tested by using cloned genes (sequences shown in Fig. 1b) as templates, except the BB form, which has not yet been found to occur in nature. The primers that differed in one or two bases at the 3′ terminus, discriminated the plasmids very efficiently, similar to the result for the coding region differences (Fig. 2). The melting curves showed the primer sets amplified a single fragment (data not shown). Sequencing of genomic MnSOD from a wheat BAC library showed there were no introns in the 3′ UTR and the melting point experiment in qRT-PCR using wheat genomic DNA as templates indicated only one PCR product was formed in each reaction (data not shown). Therefore, we used qRT-PCR to determine the relative copy numbers of MnSOD 3′ UTR variants in genomic DNA of wheat lines 442 and 443. The ratio of occurrence of the AA:AB:BA forms in genomic DNA was 1.1:4.5:1. The number of copies of each

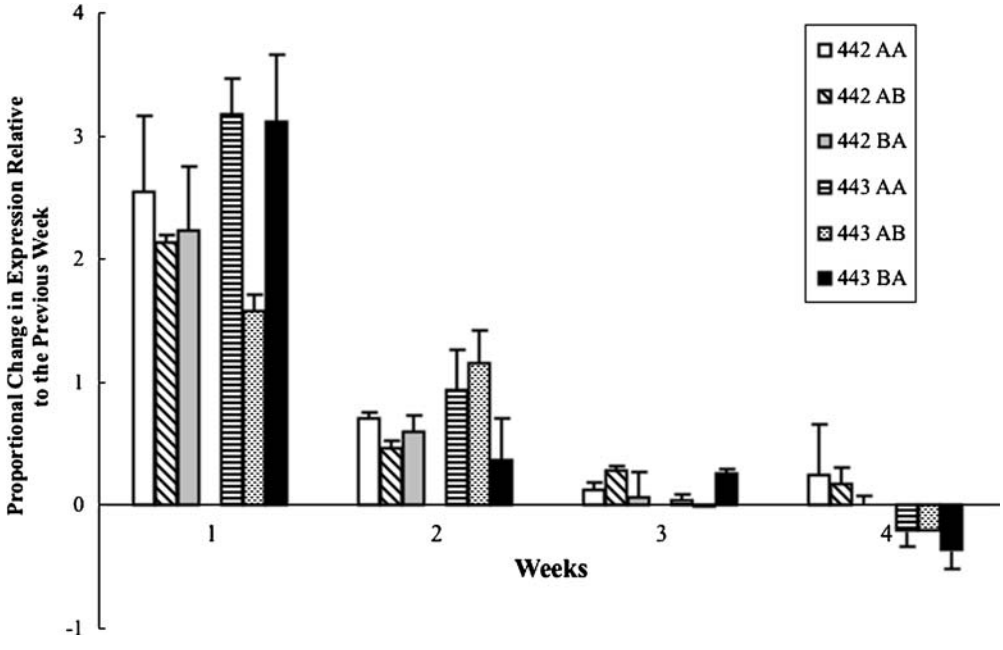



**Fig. 6** Dynamics of copy numbers of 3′ UTR variant mRNA transcripts of MnSOD in wheat NIL 442 and 443 expressed as number of copies per ng total RNA divided by the number of copies of the corresponding genes in 1 ng of total DNA (approximately mRNA copies per gene) over 4 weeks of cold acclimation. Bars represent one standard deviation unit

form per ng of DNA was used to represent the number per genome, to allow expression of mRNA transcript copies on a per-gene basis.

All 3′ UTR variants except BB were expressed at 20°C (Fig. 5). In NIL 442, AA, AB, and BA variants increased expression steadily over 4 weeks of cold acclimation. However, in NIL 443, the expression levels of variant forms AA, AB, and BA increased for the first 3 weeks of cold acclimation, then decreased from 3 to 4 weeks of cold acclimation (Fig. 5). The expression level of the AB variant in both NIL 442 and 443 was the highest among the 3' UTR variants studied, followed by AA, then BA variants. The BB form was not detected in any of the samples assayed.

The 3′ UTR does not contribute to the structure of the holoenzyme, but may contribute to the stability of the RNA molecule following transcription. The thermostabilities of the secondary structure of the three 3′ UTR variants we studied here were calculated with online software (http://www.genebee.msu.su/services/rna2_reduced.html) (Brodsky et al. 1992, 1995). The 3′ UTRs comprising the AA, AB, and BA forms had stability energies of −30, −25.1, and −35.6 kcal/mol, respectively. These values did not appear to be strongly related to the expression level (Fig. 5). The BA form had the lowest level of expression and the greatest thermostability, while the greatest level of expression was seen with the AB form (Fig. 5), which had an intermediate level of 2° structure thermostability.

Expressing the numbers of copies of 3′ UTR variants on a per gene copy number basis revealed that the AA form (with no gaps) was expressed with more than two times as many copies per gene as the AB or BA form at each of the time points (Fig. 5). The changes in these per gene copy numbers after each week of cold acclimation indicated that the AB form was not as responsive to the onset of cold temperature as the AA or BA form; the difference was significant only in the spring wheat NIL (Fig. 6). Also, the expression level of each of the forms increased over the 4-week period in the winter wheat NIL, but decreased after 3 weeks in the spring wheat NIL (Fig. 6).

## Discussion

Others have found that some gene forms are more responsive to a specific stress than others, such as dehydrin genes in the response of barley to freezing stress (Zhu et al. 2000), and alternative oxidase genes involved in soybean response to several stress factors (Djajanegara et al. 2002). Several studies have found that the 3′ UTR plays a role in transcript accumulation and post-transcriptional stability (Newman et al. 1993; Gil and Green 1996; Chan and Yu 1998; Rott et al. 1998; Komine et al. 2002). We found that the level of transcript accumulation differed for MnSOD genes with different 3′ UTRs, and that accumulation also differed in spring wheat versus winter wheat NILs. Comparison of relative numbers of copies of transcripts showed the expression level of the 3′ UTR form with no gaps (AA form) was more than twice that of the forms with gaps (AB and BA) per gene copy at each of the observed time points during cold acclimation (Fig. 5). These observations suggested that the nucleotide bases found in the AA form but not the AB or BA forms (Fig. 1b), impacted the accumulation of mRNA transcripts of the various forms of the MnSOD gene. The fact that the BB form (occurrence of both gaps) has not been reported and was not detected by PCR in this study, further suggests these bases play a significant role in functionality of the MnSOD genes.

Three species believed to be ancestral to wheat, *T. monococcum*, *A. speltoides*, and *A. tauschii*, were shown to contain paralogous forms of the MnSOD gene, suggesting that the variation seen in hexaploid wheat consisted of paralogous forms, at least some of which originated prior to the allopolyploidization events that led to the formation of hexaploid wheat, as well as the homeologous forms originating from the component genomes.

Quantification of expression levels of the amino acid position 166 variants all indicated a significant increase of expression after 1 week of cold exposure, followed by essentially constant levels of expression through the fourth week in both NIL 442 and NIL 443 (Fig. 4). A continuous increase of expression of the 3′ UTR form AB was seen through the 4-week period in NIL 442 (Fig. 5), suggesting that the 3′ UTR variation assay detected forms of the gene that were not detected by the primers used in the amino acid 166 position quantification. While a similar pattern was seen in NIL 443, a significant decrease was seen by the end of the fourth week. Hence, it appears that the levels of expression within the MnSOD gene family in wheat are quite variable, and various forms of the gene continue to express longer during cold acclimation of NIL 442 (winter wheat) compared to the corresponding spring wheat NIL 443. NIL 442 develops much more cold tolerance than NIL 443 (Storlie et al. 1998); this observation, considered with the results reported here, suggests that some forms of the MnSOD gene may contribute more strongly than others to the development of cold acclimation.

## References


Allen RD (1995) Dissection of oxidative stress tolerance using transgenic plants. Plant Physiol 107:1049–1054

Baek K-H, Skinner DZ (2003) Alteration of antioxidant enzyme gene expression during cold acclimation of near-isogenic wheat lines. Plant Sci 165:1221–1227

Bowler C, Slooten L, Vandenbranden S, De Rycke R, Botterman J, Sybesma C, Van Montague M, Inzé D (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. EMBO J 10:1723–1732

Bowler C, Montagu MV, Inzé D (1992) Superoxide dismutase and stress tolerance. Annu Rev Plant Physiol Plant Mol Biol 43:83–116

Breusegem F, van Slooten L, Stassart JM, Botterman J, Moens T, Montagu M, van Inze D (1999) Effects of overproduction of tobacco MnSOD in maize chloroplasts on foliar tolerance to cold and oxidative stress. J Exp Bot 50:71–78

Brodsky LI, Vasiliev AV, Kalaidzidis YaL, Osipov YuS, Tatuzov RL, Feranchuk SI (1992) GeneBee: The program package for biopolymer structure analysis. Dimacs 8:127–139

Brodsky LI, Ivanov VV, Kalai dzidis YaL, Leontovich AM, Nikolaev VK, Feranchuk SI, Drachev VA (1995) GeneBee-NET: Internet-based server for analyzing biopolymers structure. Biochemistry 60:923–928

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193

Carlioz A, Touati D (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: Is superoxide dismutase necessary for aerobic life? EMBO J 5:623–630

Chan MT, Yu SM (1998) The 3′-untranslated region of a rice alpha-amylase gene functions as a sugar-dependent mRNA stability determinant. Proc Nat Acad Sci USA 95:6543–6547

Clare DA, Rabinowitch HD, Fridovich I (1984) Superoxide dismutase and chilling injury in *Chlorella ellipsoidea*. Arch Biochem Biophys 231:158–168

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucl Acids Res 16(22):10881–10890

Dellaporta SL, Wood J, Hicks JB (1985) Maize DNA miniprep. In: Malberg R, Messing J, Sussex I (eds) Molecular biology of plants. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, p 3637

Djajanegara I, Finnegan PM, Mathieu C, McCabe T, Whelan J, Day DA (2002) Regulation of alternative oxidase gene expression in soybean. Plant Mol Biol 50:735–742

Fink RC, Scandalios JG (2002) Molecular evolution and structure–function relationships of the superoxide dismutase gene families in angiosperms and their relationship to other eukaryotic and prokaryotic superoxide dismutases. Arch Biochem Biophys 399:19–36

Fridovich I (1991) Molecular oxygen; friend and foe. In: Pell EJ, Steffen KL (eds) Active oxygen/oxidative stress and plant metabolism. American Society of Plant Physiologists, Rockville, pp 1–5

Fucci L, Oliver CN, Coon MJ, Stadtman ER (1983) Inactivation of metabolic enzymes by mixed-function oxidation reaction: Possible implication in protein turnover and ageing. Proc Natl Acad Sci USA 80:1521–1525

Gachon C, Mingam A, Charrier B (2004) Real-time PCR: What releveance to plant studies? J Exp Bot 55:1445–1454

Gil P, Green PJ (1996) Multiple regions of the Arabidopsis SAUR-AC1 gene control transcript abundance: The 3′ untranslated region functions as an mRNA instability determinant. EMBO J 15:1678–1686

Halliwell B, Gutteridge JMC (1999) Free radicals in biology and medicine, 3rd edn. Oxford University Press, New York

Karsai A, Müller S, Platz S, Hauser M (2002) Evaluation of a homemade SYBR(r) Green I reaction mixture for real-time PCR quantification of gene expression. BioTech 32:790–796

Kendall EJ, McKersie BB (1989) Free radical and freezing injury to cell membranes of winter wheat. Physiol Plant 76:86–94

Komine Y, Kikis E, Schuster G, Stern D (2002) Evidence for in vivo modulation of chloroplast RNA stability by 3-UTR homopolymeric tails in *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 99:4085–4090

McKersie BD, Chen Y, De Beus M, Bowley SR, Bowler C, Inze D, D'Halluin K, Botterman J (1993) Superoxide dismutase enhances tolerance of freezing stress in transgenic alfalfa (*Medicago sativa* L.). Plant Physiol 103:1155–1163

Miao Z, Gaynor JJ (1993) Molecular cloning, characterization and expression of Mn-superoxide dismutase from the rubber tree (*Hevea brasiliensis*). Plant Mol Biol 23:267–277

Monk LS, Fagerstedt KV, Crawford RMM (1989) Oxygen toxicity and superoxide dismutase as an antioxidant in physiological stress. Physiol Plant 76:456–459

Newman TC, Ohme-Takagi M, Taylor CB, Green PJ (1993) DST sequences, highly conserved among plant SAUR genes, target reporter transcripts for rapid decay in tobacco. Plant Cell 5:701–714

Rott R, Liveanu V, Drager R, Stern D, Schuster G (1998) Sequence and structure of the 3' untranslated region are important determinants for accumulation and stability of chloroplast mRNA. Plant Mol Biol 36:307–314

Sato I, Zu J, Nishikawa S, Kashimura N (1993) Depolymerization of hyaluronic acid by D-fructose 6-phosphate. Biosci Biotechnol Biochem 57:2005–2009

Storlie EW, Allan RE, Walker-Simmons MK (1998) Effect of the Vrn1-Fr1 interval on cold hardiness levels in near isogenic wheat lines. Crop Sci 38:483–488

Wu G, Shortt BJ, Lawrence EB, Fitzsimmons JLKC, Levine EB, Raskin I, Shah DM (1997) Activation of host defense mechanisms by elevated production of $H_2O_2$ in transgenic plants. Plant Physiol 115:427–435

Wu G, Wilen RW, Robertson AJ, Gusta LV (1999) Isolation, chromosome localization, and differential expression of mitochondrial manganese superoxide dismutase and chloroplastic copper/zinc superoxide dismutase genes in wheat. Plant Physiol 120:513–520

Zelko IN, Mariani TJ, Folz RJ (2002) Superoxide dismutase multigene family: A comparison of CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic Biol Med 33:337–349

Zhu B, Choi D-W, Close TJ (2000) Expression of the barley dehydrin multigene family and the development of freezing tolerance. Mol Gen Genet 264:145–153

Zhu D, Scandalios JG (1993) Maize mitochondrial manganese superoxide dismutases are encoded by a differentially expressed multigene family. Proc Natl Acad Sci USA 90:9310–9314